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TITLE: Mechanisms of Transforming Growth Factor Beta-Receptor II Loss in Breast Neoplasia

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13. ABSTRACT ***(Maximum 200 Words)***

Epidemiologic studies show that most human invasive breast carcinomas arise from preexisting benign lesions. Usual hyperplasia without atypia has a two fold elevation in risk for subsequent invasive breast cancer compared with women without proliferative disease. In order to identify the women at high risk,_ knowledge of molecular markers of breast neoplastic progression is needed Transforming growth factor-betas are important growth suppressing factors in normal breast epithelium. and their activity is mediated by specific receptors, including transforming growth factor beta receptor II (TGFbRII). Most normal breast epithelium express high levels ofTGFbR II. Loss of expression ofTGFbR II is related to cell proliferation and tumor progression. A recent study showed that reduced levels of TGFbR II in epithelial hyperplasia lacking atypia added an additional risk of invasive breast cancer. The molecular alterations responsible for the development and progression of proliferative breast diseases without atypia are not understood. We hypothesized that loss of TGFbR II expression in usual hyperplasia identifies a subset of women at increased risk of breast cancer. To test this hypothesis we have established a repository of African American breast biopsies from Meharry Medical College and Metropolitan Nashville General Hospital from 1960-1995 (2.255 cases). Histologically confirmed cases of usual hyperplasia without atypia lesions in African American women are selected and immunohistochemically stained for TGFbR II and Ki-67(MIB-l) to determine the loss of expression and proliferation. The percentage of positive cells in hyperplastic lesions are assessed as less than 25%, 25%-75 %, and greater than 75% for TGFbR II. A cell is considered positive if there is any nuclear staining for MIB-l. In usual hyperplasia cases from the Nashville Breast Study Cohort and African American repository. lesions with less than 25% immunoreactivity for TGFbR II gene expression, will be microdissected and the DNA will be isoloated from paraffin-embedded tissue to determine if the decreased expression is due to loss of heterozygosity (LOH) at the TGFbR II locus. LOH at the locus will be assayed using microsatellite markers D3S1567, D3S1609, and D3S3547.

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Introduction

Transforming growth factor betas are important growth suppressing factors in normal breast epithelium, and their activity is mediated by specific receptors, including transforming growth factor beta-receptor II (TGFβRII). Most normal breast epithelium expresses high levels of TGFβRII. Loss of expression of TGFβRII is related to cell proliferation and tumor progression. A recent study showed that reduced levels of TGFβRII in epithelial hyperplasia lacking atypia added an additional risk of invasive breast cancer to women with this type of lesion. The molecular alterations responsible for the development and progression of proliferative breast diseases without atypia (usual hyperplasia) are not well understood. Prior studies would suggest that, in some cases of usual hyperplasia, the loss of TGFβRII expression may indicate an important subset of hyperplasias at increased risk for malignant progression1.

Body

Phase I Project Startup and Parameter Development: (Months 1-6)

Meet with investigator at VUMC

Dr. Roy Jensen and Dr. Digna Forbes had a total of 49 meetings starting from April 7, 2000 through December 18, 2002 at VUMC(Palm Pilot entries) to discuss the technical difficulties and progression of research.

Begin training in LCM, DNA and RNA nucleic acid extraction, PCR, LOH and Methylation

Initial training began with an in-service workshop given by the sales representative of Arcturus in the use and maintenance of the Laser Capture Microdissection Microscope(LCM) on 12-18-00 at VUMC. Hands on training sessions was conducted by Dr. Jensen and Tissue Resource Laboratory Manager, Kim Newsom Johnson on the preparation of paraffin embedded breast tissue slides for microdissection and using the LCM to microdissect normal breast tissue; a total of 69 cases were microdissected. I also worked in the Laboratory of Dr. Jeffrey Holt with Laboratory Manager, Patrice Brown approximately three afternoons a week. Research steps involved using the epithelial cells microdissected from normal breast and extracting the DNA using the LCM/DNA Extraction Kit. Performed polymerase chain reaction using microsatellite markers for LOH analysis at the TGF-beta receptor II locus, using the Epicentre Failsafe premix kit followed by agarose and acrylamide gel electrophoresis and autoradiography. Dr. Forbes was certified at Meharry and Vanderbilt to use radioactive material after a two-day in-service course and examination.

Attend AACR Conference

Additionally, there was training in Southern Analysis for DNA methylation and the attendance of the AACR Conference on Cancer & Chromosomal Organization, Epigenetics of Cancer (October 17-21,2001); with the information learned and discussed at the meeting, Dr Jensen and I decided that methylation at the TGFβRII locus does not play a role in its function and thus it was decided this specific aim would not be pursued.

Phase II Project Development and Transition From VUMC to MMC: (Months 7-12)

Selection of case patients and controls of African American breast biopsies from MetroGeneral Hospital and Meharry Medical College

Laboratory space was provided by Meharry Medical College and major equipment, glassware and supplies were purchased. The cases from Meharry Medical College and Metro General Hospital from 1960 to 1995 were not on computers, so the breast biopsies had to be retrieved from daily surgical logbooks and bound surgical pathology books. Once the breast biopsy cases were identified they were then grouped according to race, diagnosis and then de-identified. These cases were then typed into a spreadsheet, and the spreadsheet was downloaded into STATA software.

Hire Research Assistant

A research assistant was tasked with assisting Dr. Forbes in pulling slides/ blocks and entering data into the computer. All the slides for the benign breast cases were pulled from Meharry/MetroGeneral files and stored in the lab of Dr. Forbes at Meharry Medical College.

Immunohistochemical evaluation of TGF-beta receptor II

The benign cases were microscopically reviewed by Dr. Forbes for lesions that met the histologic criteria of usual hyperplasia without atypia, then corresponding tissue blocks were located and pulled. Metro General tissue blocks were stored in sub-optimal conditions off site, however the hospital administration agreed to move all the blocks to the main hospital for proper storage. Of the 2,463 cases identified and microscopically reviewed, a repository of African American breast biopsies from Meharry Medical College and Metropolitan Nashville General Hospital from 1960-1995 was established. In our study histologically confirmed cases of usual hyperplasia without atypia lesions in African American women were selected from Meharry Medical College between 1970-1994 and Metropolitan Nashville General Hospital from 1976-1994. The tissue blocks from 1960-1969 from Meharry were not found, the slides were available. The tissue blocks and the slides from 1960-1975 were not found at Metro General. A total of 180 microscopically reviewed cases met the histologic criteria of usual hyperplasia without atypia. In each of the 180 cases, sequential 5μm sections from formalin fixed, paraffin embedded tissue of diagnostic biopsies were cut. One section from each specimen was stained with hematoxylin-eosin (H&E) to verify the presence of epithelial hyperplasia. Sequential step sections were immunohistochemically stained with TGFβRII and used for Laser Capture Microdisecction (LCM). Immunostaining was performed on a Ventana ES-automated immunostainer (Ventana, Tucson, AZ) by use of an affinity-purified rabbit polyclonal antibody (C-16: Santa Cruz, Biotechnology, Santa Cruz, CA) raised against human TGFβRII. This antibody recognizes sequences corresponding to amino acids 550-565 located at the C-terminal region of the TGFβRII protein. A concentration of 2μg/ml by use of Protease I (Ventana) pretreatment is applied to all specimens. Reduction mammoplasty specimen was used as a positive control for TGFβRII antibody and epithelia of normal ducts and lobular units adjacent to usual hyperplasia was used as internal controls for each specimen. A semiquantitative approach to distinguish levels of expression among TGFβRII positive specimen was utilized as described by Gobbi et al.(2) An initial review of all 180 cases immunostained slides was conducted to choose cut-off points and a scoring system that permitted reproducibility of the semiquantitative analysis. Two pathologists (R.A.J. and D.S.F.) assessed each case independently, and discrepancies were resolved by simultaneous viewing and consultation at our semi-weekly sessions. The staining is positive when the cytoplasm of normal ductal epithelium or lobular units stains

for TGFβRII. The percentage of positive cells in usual hyperplasia is assessed as less than 25%, 25%-75%, and greater than 75%. Homogeneous pattern is classified as more than 95% of positively stained cells with a similar staining intensity; otherwise it is classified as a heterogeneous pattern. The intensity of positive cells staining is grouped into weak, moderate, and strong categories by making a comparison with positive epithelial cells of normal ducts and lobular units within the same biopsy specimen. (1,2,4). Of the 180 cases reviewed for immunostaining, thirteen cases were identified as staining less than 25% which is negative, with an age range from 16-64 years.

Immunohistochemical evaluation with Ki-67

These cases were immunohistochemically stained for Ki-67 (MIB-1) to determine if the cell with a loss of TGFβRII staining were positive for MIB-1 that corresponds with an increase in cell proliferation. Immunostaining was performed on a Dako automated immunostainer (Carpinteria, CA). In immunoprecipitation and immunoblotting experiments the antibody recognizes two cell cycle associated bands of 345kDa and 395kDa identical with the pattern of the Ki-67 antigen. (5,6) The Dako antibody recognizes an epitope in the nuclei of proliferating cells and binds to a formalin resistant epitope of the Ki-67 antigen. We identified areas negative for TGFβRII staining and matched up corresponding areas of MIB-1 scoring on the step section on the thirteen cases that stained less than 25% for TGFβRII . All sections were examined with a 40X objective and a minimum of 500 cells was counted for each case. A cell is considered positive if there is any nuclear staining for MIB-1. An MIB-1 score is identified as the number of cells with nuclear immunostaining divided by the total number of cells counted from the usual hyperplasia lesion. There were three cases of the thirteen that were positive for MIB-1.

LOH Analysis of TGFβRII

LOH Analysis of TGFβRII on the thirteen cases of usual hyperplasia cases from the African American Repository with less than 25% immunoreactivity for TGFβRII gene expression, LCM (Arcturus, Mountainview, California) was used to isolate cells and subsequently DNA from the specimen using the Arcturus LCM/DNA Extraction Kit. LOH at the TGFβRII locus was assayed using microsatellite markers D3S1567, D3S1609, and D3S3547 (7). Polymerase chain reaction (PCR) was performed in 25μl volumes using 12μl of Failsafe Tm PCR 2X Premixes A, B, G, H or I (Epicentre Corp., Madison WI), 6.0ul sterile water, 200 nm of each primer, 1.25U Failsafe PCR Enzyme Mix, 50-100ng DNA sample. A 40-cycle amplification is done in a thermal cycler (GeneAmp PCR System 2700). Unfortunately LOH was not successful due to poor preservation of DNA.

Phase III Analysis and interpretation of data gathered during phase II(Months 13-18)

A total of 180 cases met the histologic criteria of usual hyperplasia without atypia, of these 155 cases stained with greater than 75% TGFβRII (86%), 12 cases stained with 25%-75% TGFβRII (7%) and 13 cases stained with less than 25% TGFβRII (7%). **The proportion of TGFβRII staining levels varies significantly between black and white EHLA patients (P<0.00005, chi squared test of homogeneity of odds).Black women are less likely to have reduced TGFβRII staining levels than white women(Odds ratio=0.34 P=0.0002). The racial difference is greatest for staining reductions in the 25-75% range. The odds ratio for 25-75% staining vs. normal staining in blacks**

relative to whites is 0.208(P<0.00005). However the corresponding odds ratio for <25% staining is only 0.817(P=0.67). Hence, we do not see a trend of decreasing odds ratios with decreasing levels of expression in blacks compared to whites. Nevertheless, the P value for any reductions in staining level is highly significant. (This result is consistent with black women having a lower incidence of breast cancer than white women). The 13 cases stained with less than 25% TGFβRII, were subsequently stained with MIB-1, 10 cases were negative for cell proliferation and three cases were positive for cell proliferation. LOH was not successful in the 13 cases due to the poor preservation of DNA

Key Research Accomplishments

Successfully established unique cohort of breast biopsy patients in the African-American community from Meharry Medical College and Metropolitan Nashville General Hospital 1960-1995(2,463 cases).

LCM and isolation of DNA from paraffin embedded tissues.

Established conditions for TGFβRII LOH assay.

Immunohistochemical evaluation of TGFβRII and MIB-1.

MMC laboratory established with Research Assistant.

Reportable Outcomes

Preliminary data utilized to develop a project for the recently funded National Cancer Institute Vanderbilt-Meharry Specialized Program of Research Excellence in Breast Cancer.

Abstract presentation at the DOD Era of Hope Breast Cancer Meeting Sept 2002.

2002 AACR -HBCU Faculty Scholar Award in Cancer Research

Conclusions

A total of 180 cases met the histologic criteria of usual hyperplasia without atypia(EHLA), of these 155 cases stained with greater than 75% TGFβRII (86%), 12 cases stained with 25%-75% TGFβRII (7%) and 13 cases stained with less than 25% TGFβRII (7%). **The proportion of TGFβRII staining levels varies significantly between black and white EHLA patients (P<0.00005, chi squared test of homogeneity of odds).Black women are less likely to have reduced TGFβRII staining levels than white women(Odds ratio=0.34 P=0.0002). The racial difference is greatest for staining reductions in the 25-75% range. The odds ratio for 25-75% staining vs. normal staining in blacks relative to whites is 0.208(P<0.00005). However the corresponding odds ratio for <25% staining is only 0.817(P=0.67). Hence, we do not see a trend of decreasing odds ratios with decreasing levels of expression in blacks compared to whites. Nevertheless, the P value for any reductions in staining level is highly significant. (This result is consistent with black women having a lower incidence of breast cancer than white women).** The 13 cases stained with less than 25% TGFβRII, were subsequently stained with MIB-1, 10 cases were negative for cell proliferation and three cases were positive for cell proliferation. LOH was not successful in the 13 cases due to the poor preservation of DNA. Because of the terms of our IRB approval we were not allowed to obtain clinical follow up or menopausal status. At a future date we intend to seek IRB approval to see if the 13 cases identified with a loss of TGFβRII staining and positive for MIB-1 staining have an increased risk in developing breast cancer.

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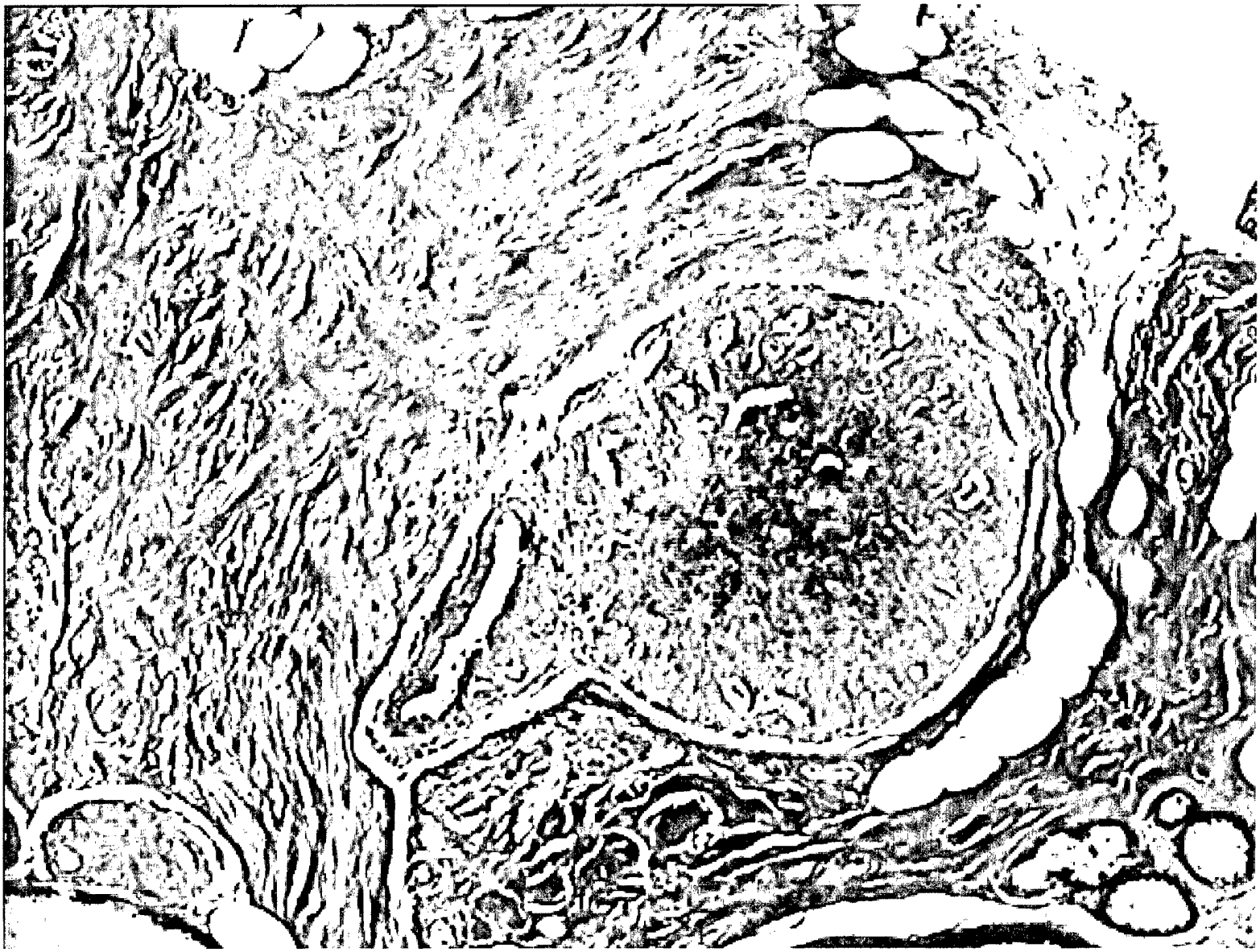
DR JENSEN'S PALM PILOT ENTRIES OF MEETING DATES WITH DR FORBES

2000	2001	2002	2003
04/07/00	01/03/01	01/15/02	02/05/03
06/06/00	01/17/01	03/11/02	
06/12/00	01/24/01	03/19/02	
06/27/00	02/07/01	03/27/02	
07/11/00	02/21/01	04/03/02	
07/13/00	03/14/01	05/15/02	
08/09/00	03/21/01	05/24/02	
09/13/00	03/28/01	06/05/02	
09/27/00	04/04/01	06/12/02	
10/28/00	04/11/01	06/26/02	
	04/25/01	08/07/02	
	05/18/01	08/21/02	
	05/23/01	09/04/02	
	07/25/01	09/11/02	
	08/01/01	09/16/02	
	08/30/01	11/06/02	
	09/26/01	11/20/02	
	11/14/01	12/18/02	
	11/16/01		
	12/07/01		
	12/17/01		

LCM TRAINING DOCUMENTATION

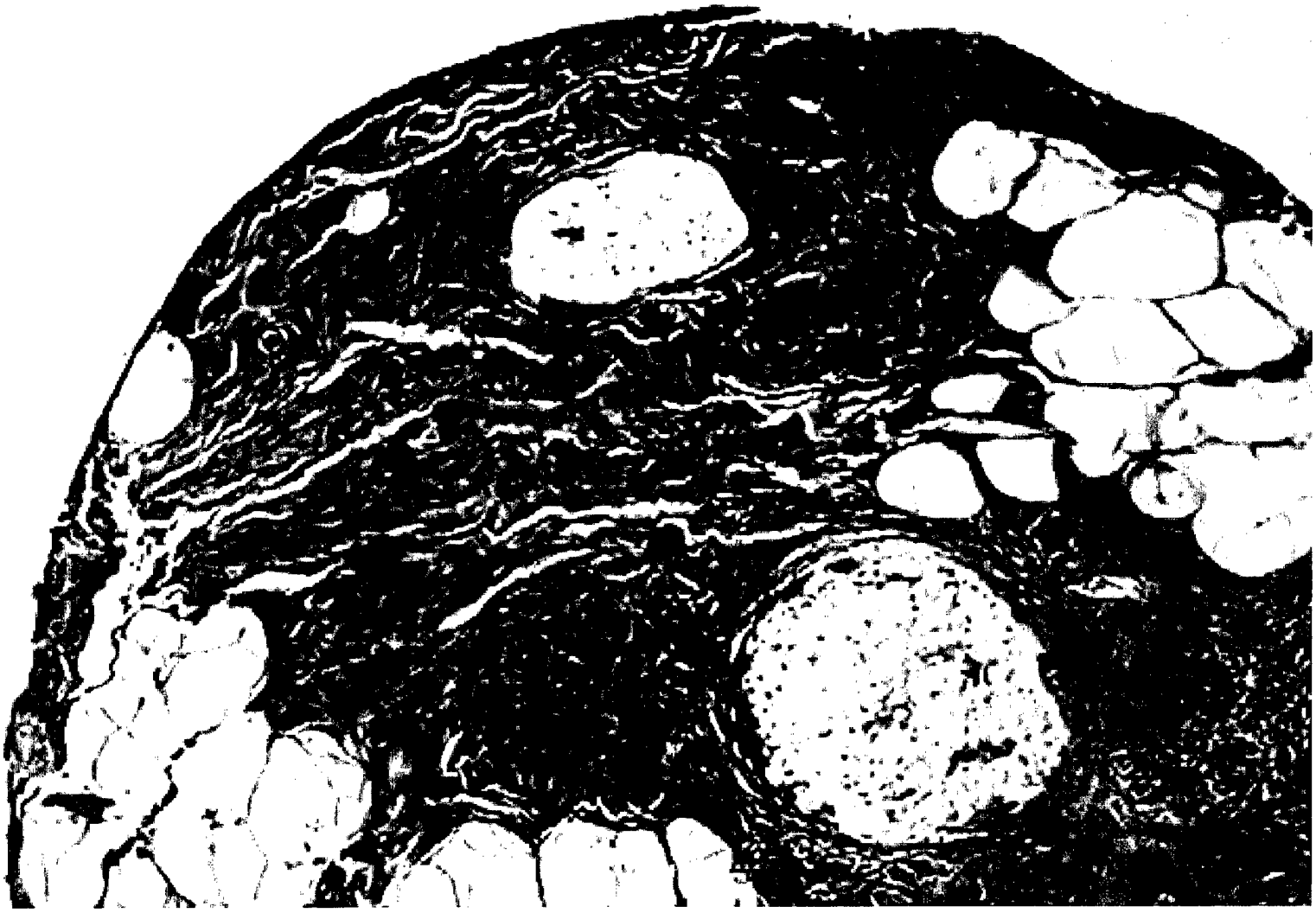
PICTURES TAKEN C LCM



H+E Section of BREAST TISSUE with EHLA

SAMPLE

LCM TRAINING DOCUMENTATION



H+E Section After LCM - microdissection after cells have been removed

SAMPLE

Breast Epithelial cells on LCM cap after microdissection DNA extracted from CAP FOR PCR analysis

SAMPLE

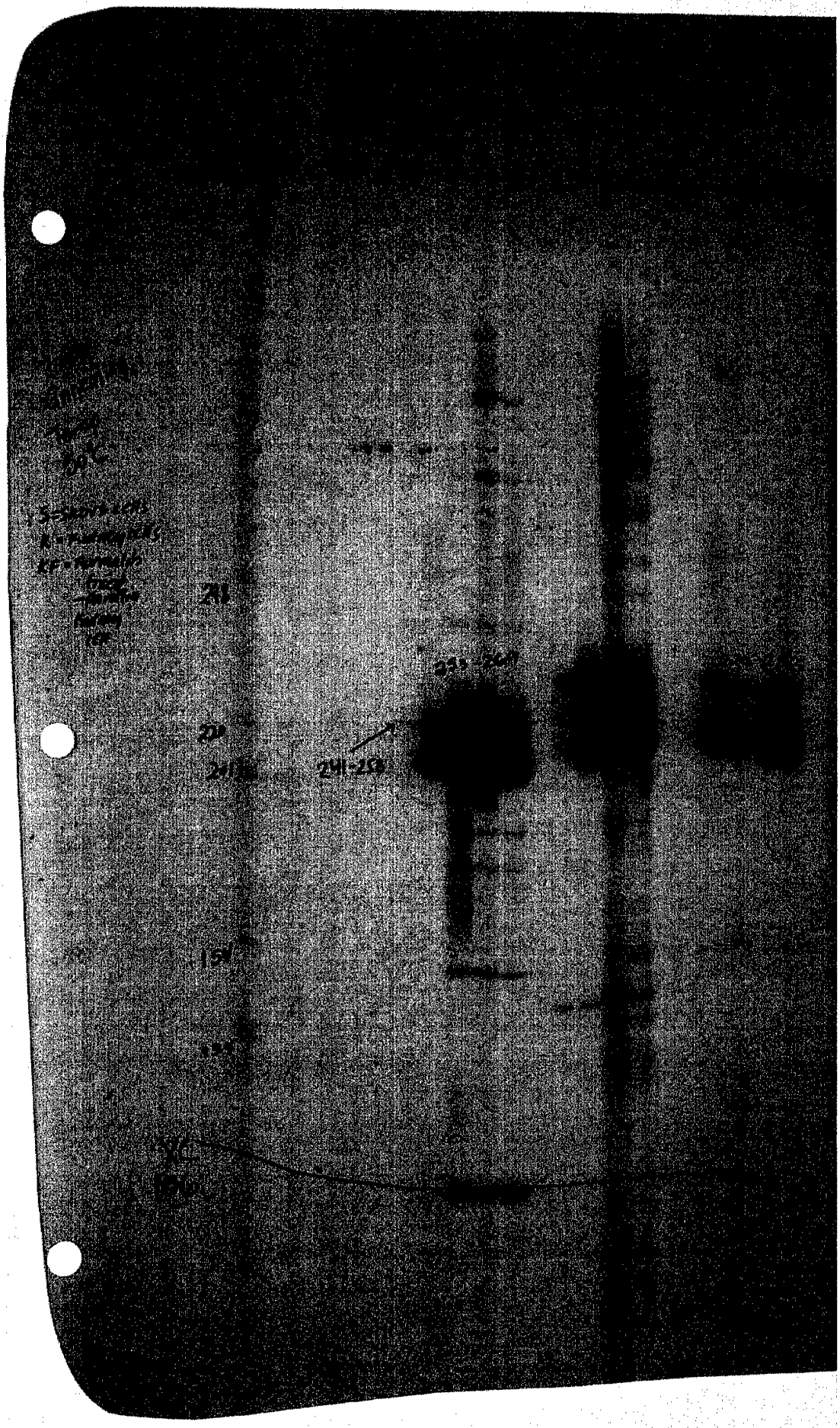
S-77-1697-1_text

DATA GENERATED FOR EACH CASE MICRO DISSECTED

PixCell Slide Dissection Data

Instrument Serial Number: 0103
Slide Number: S-77-1697-1
Cap Lot Number: 091598E-1
Slide Notes: TGFBRII
Sample Thickness: 5.00um
Laser Spot Size: 15um
Pulse Power: 60mW
Pulse Width: 3.0ms
Threshold Voltage: 335mV
Total Pulses: 1158
Estimated Transfer per Pulse: 90%
Estimated Volume of Tissue Dissected: 9.210E-007 microns^3
Images Acquired: 4
Images Saved (includes all formats): 4

6% Acrylamide Gel



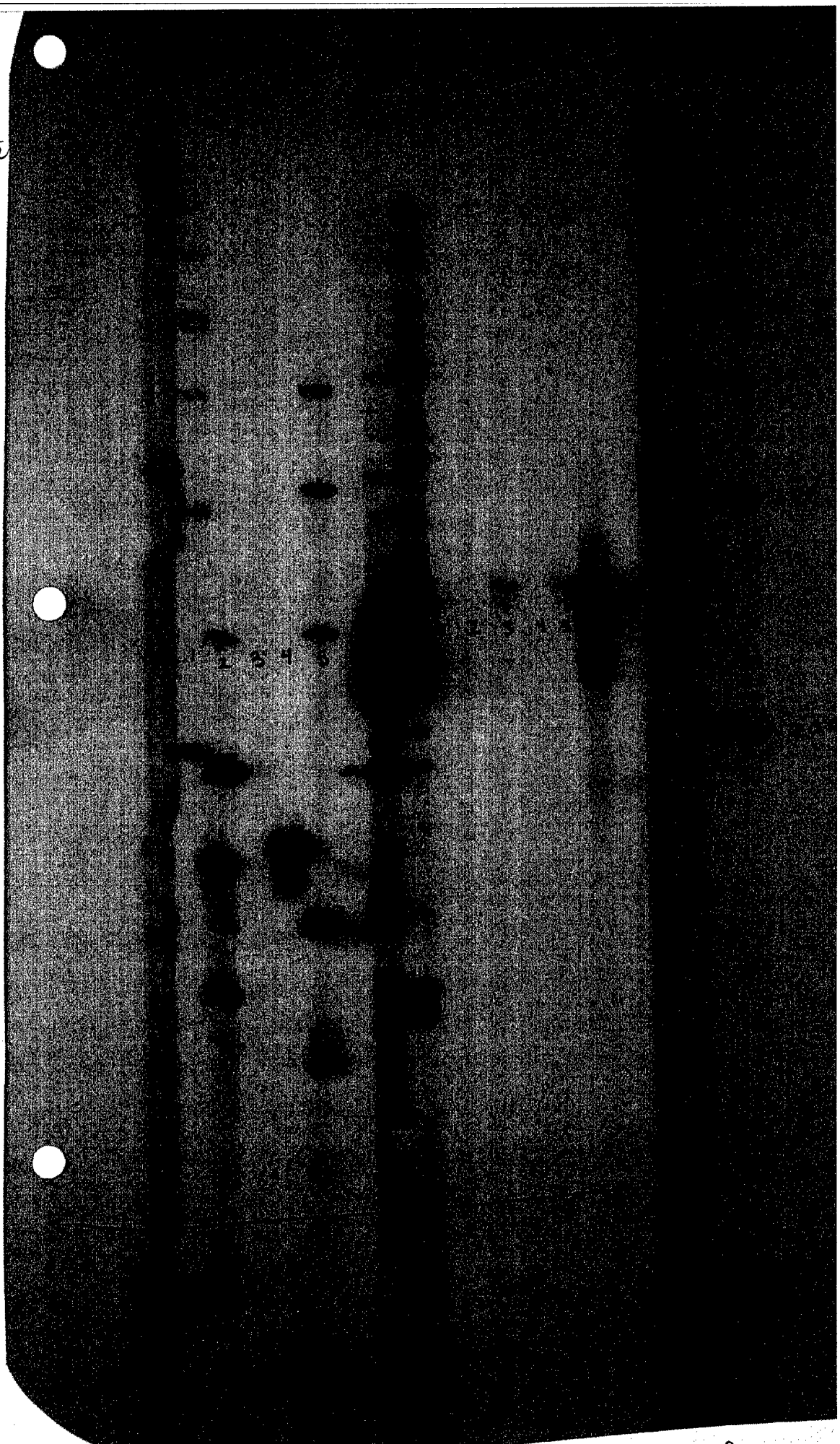
PCR analysis of kidney cells ± Oligo #1, #2, + #3 for LOH analysis
TRAINING DOCUMENTATION FOR PCR Analysis

6% Acrylamide Gel

Establish PCR procedure for LOH

#1 product 241-253

#2 product 253-269



- PCR analysis of LCM Breast Epithelial cells Oligo #1 #2 for LOH Breast Samples 2002 #6 POSITIVE control Clontech DNA

Total # of African American(A.A.) breast cases Meharry /Metro General = 2,463

Meharry Medical College (Hubbard Hospital)

1960-1994 Total cases 1999 , Total benign cases 1524, Total EHLA 180

1960-1969 Total cases 560, Total benign cases 376, No blocks found

1970-1979 Total cases 666, Total benign cases 537, Total EHLA 52

1980-1989 Total cases 599, Total benign cases 474, Total EHLA 82
1985-1986 missing reports/data

1990-1994 Total cases 174, Total benign cases137, Total EHLA 46
Hubbard Hospital closed in 1994

Metro General Hospital

1960-1994 Total AA cases 464, Total benign cases 359, EHLA 69

1960-1974 No data/ records found

1975-Records & Blocks available but racial identity not determined.

1976-1979 Total AA cases 72, Total benign cases 59 Total EHLA 7

1980-1989 Total AA cases 262, Total benign cases 201, Total EHLA 47

1990-1994 Total AA cases 130, Total benign cases 99, Total EHLA 15

Total EHLA cases 249, Total cases evaluated for TGFBRII 180

69 EHLA cases blocks were not found.

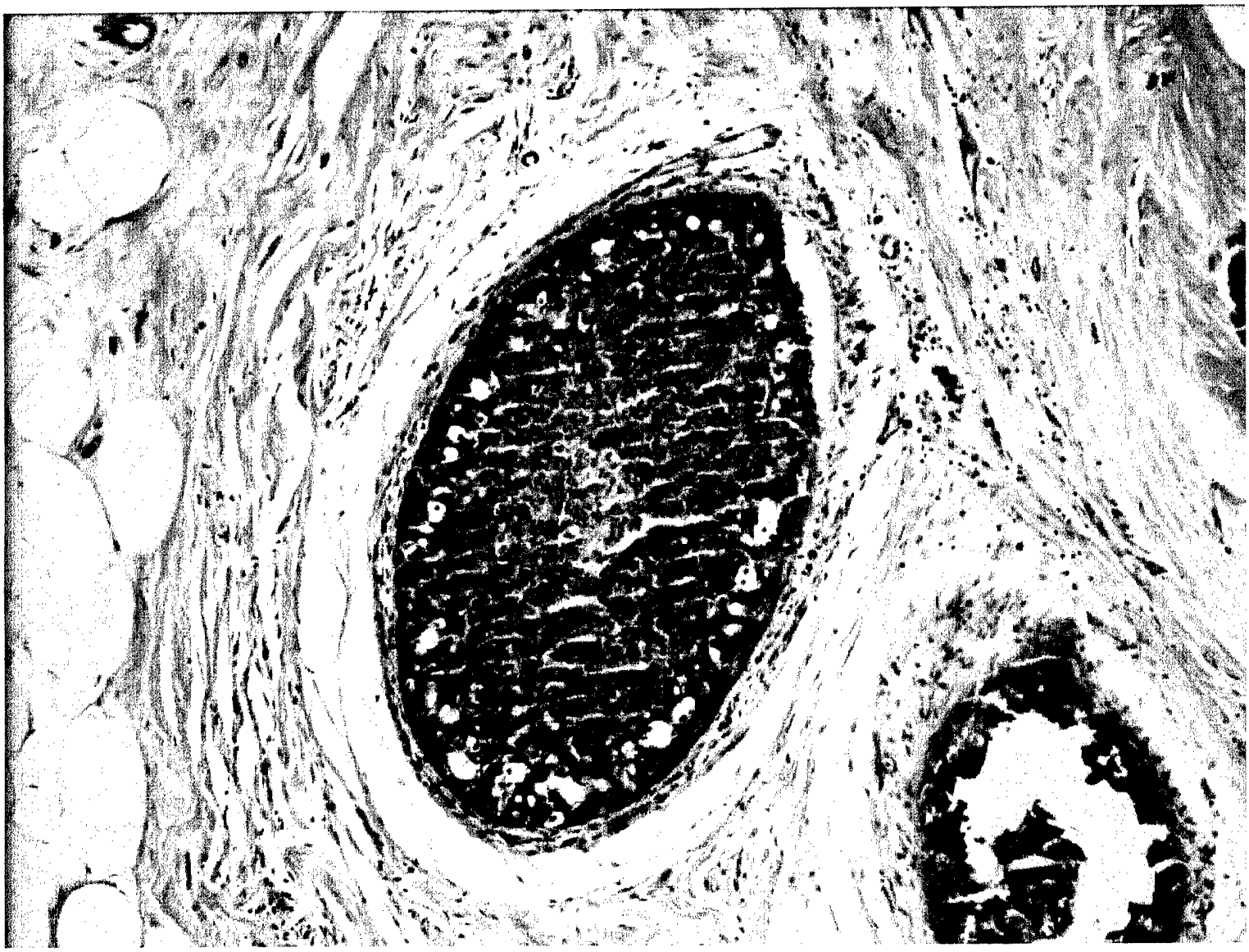


Figure 1
>75% TGFβRII staining (Objective magnification 400X)

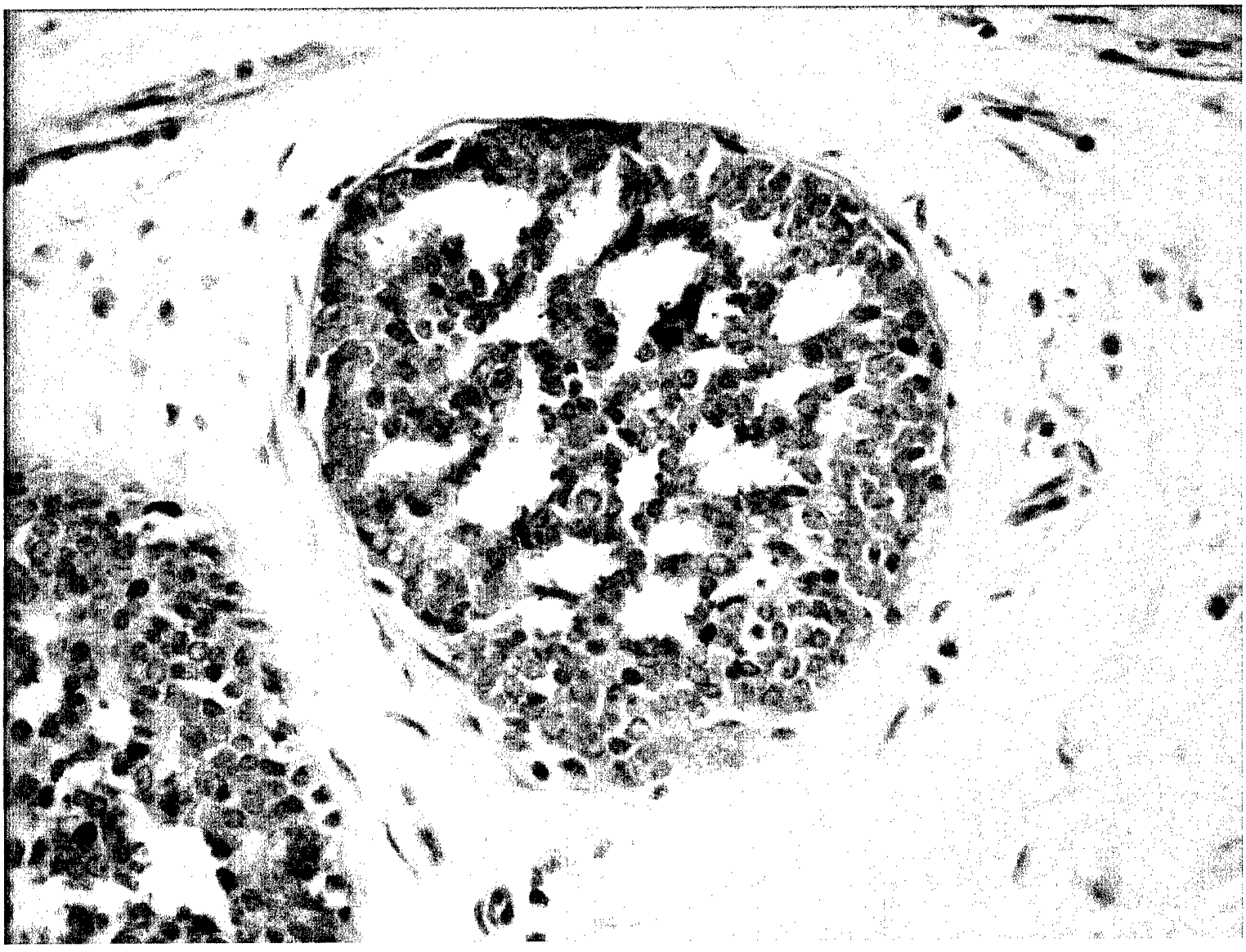


Figure 2
25%-75% TGFβRII staining (Objective magnification400X)

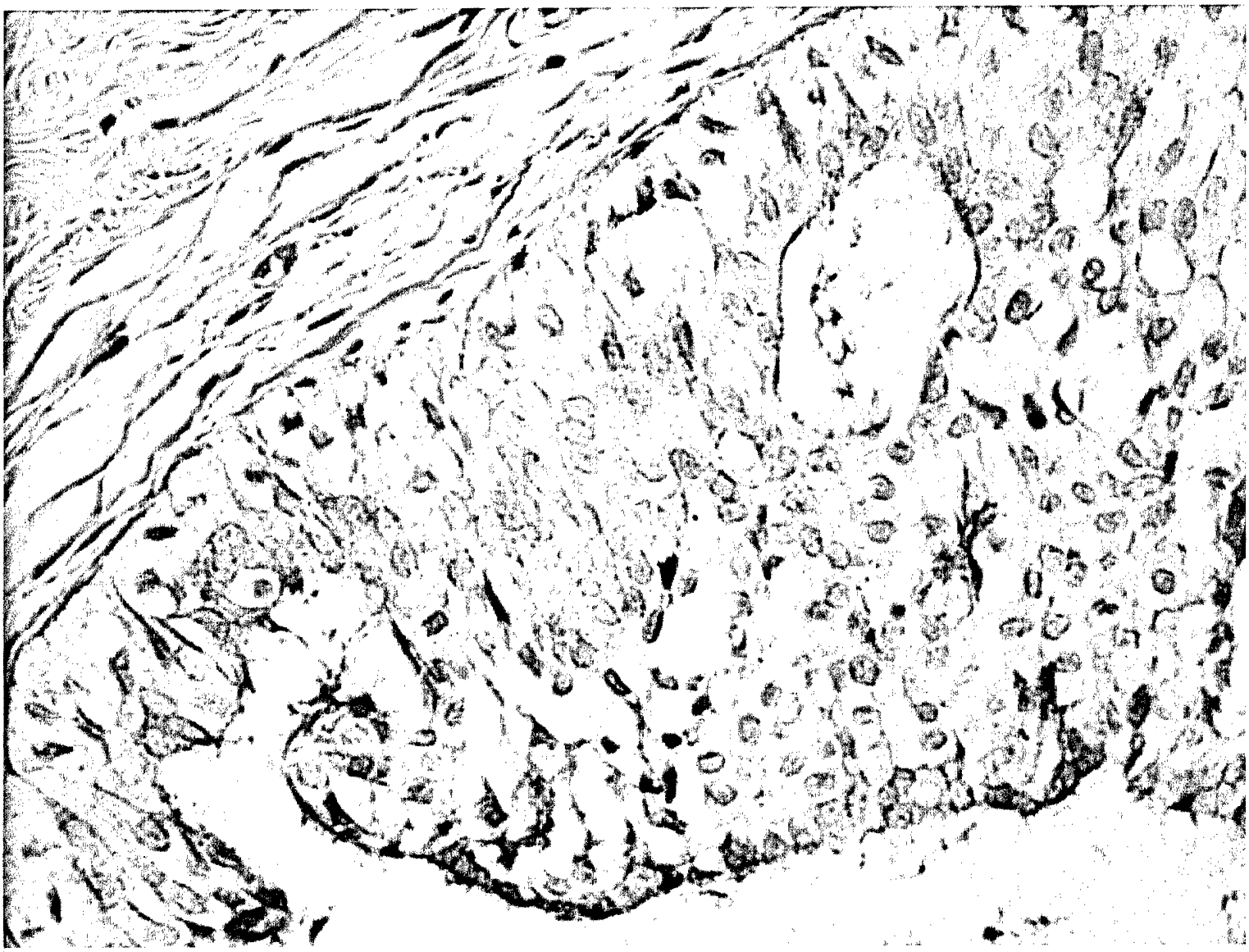
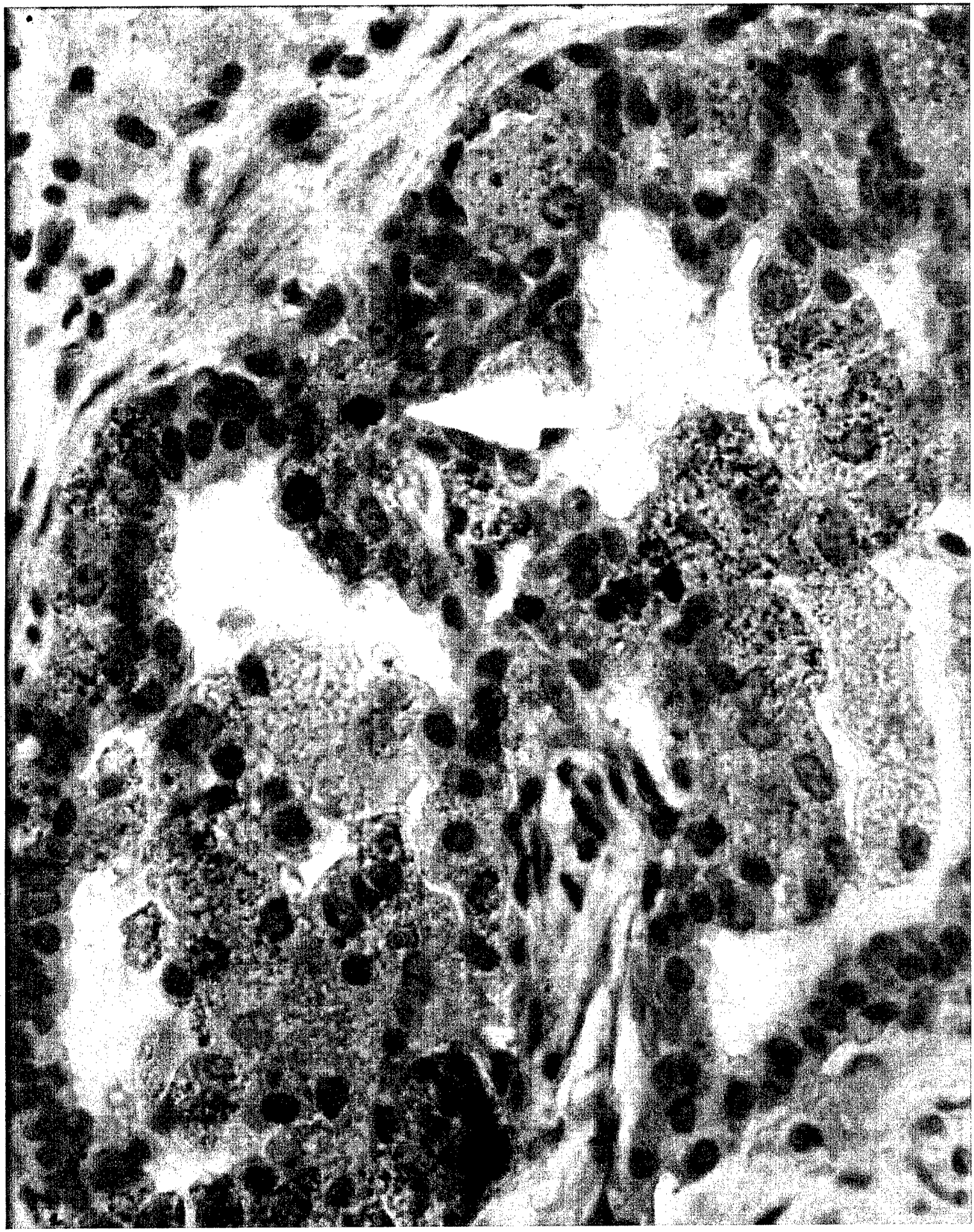


Figure 3
<25% TGFβRII Staining (Objective magnification 400 X)

Figure 4
Positive MIB-1 Staining (Objective magnification 400 X)



Results

Table 1. TGFβRII Staining in Usual Hyperplasia without Atypia

Proportion of TGFbRII positive cells	# of cases (n = 180)	Percentage
>75% staining	155	86%
25%-75% staining	12	7%
<25% staining	13	7%

STATISTICAL CALCULATIONS ON EHLA TGFbRII

```
. tabodds black r2level [fw=count]

------------------------------------------------------------------------
--
  r2level |    cases    controls      odds    [95% Conf. Interval]
          |   blacks    whites
----------+-------------------------------------------------------------
--
  > 75%  0|      155         78   1.98718      1.51380
2.60858
  25-75%  1|       12         29   0.41379      0.21116
0.81087
  < 25%  2|       13          8   1.62500      0.67353
3.92056
------------------------------------------------------------------------
--
Test of homogeneity (equal odds): chi2(2) =   20.28
                      Pr>chi2 =  0.0000

Score test for trend of odds:     chi2(1) =    6.59
                      Pr>chi2 =  0.0103


. tabulate r2level black [fw=count], all

           |      black
  r2level  |         0         1  |     Total
-----------+----------------------+----------
         0 |        78       155  |       233
         1 |        29        12  |        41
         2 |         8        13  |        21
-----------+----------------------+----------
     Total |       115       180  |       295

       Pearson chi2(2) =  20.3516   Pr = 0.000
 likelihood-ratio chi2(2) =  19.9589   Pr = 0.000
            Cramer's V =   0.2627
                 gamma =  -0.4295  ASE = 0.111
       Kendall's tau-b =  -0.1968  ASE = 0.058


. cc black r2level [fw=count] if r2level ~= 2
```

```
                                                  Proportion
           |  Exposed   Unexposed |    Total     Exposed
           |  25-75%     >75%     |
-----------+----------------------+----------------------
 Blacks  Cases |       12        155 |      167      0.0719
 Whites Controls |     29         78 |      107      0.2710
-----------+----------------------+----------------------
     Total |       41        233 |      274      0.1496
           |                      |
           |     Point estimate   | [95% Conf. Interval]
           |----------------------+----------------------
    Odds ratio |       .2082314    |  .092081    .4509508
(exact)
 Prev. frac. ex. |     .7917686    |  .5490492    .907919
(exact)
 Prev. frac. pop |     .2145915    |
           +-------------------------------------------
                     chi2(1) =    20.33  Pr>chi2 = 0.0000

. cc black r2level [fw=count] if r2level ~= 1
                                                  Proportion
           |  Exposed   Unexposed |    Total     Exposed
           |  < 25       >75%     |
-----------+----------------------+----------------------
      Cases |       13        155 |      168      0.0774
   Controls |        8         78 |       86      0.0930
-----------+----------------------+----------------------
     Total |       21        233 |      254      0.0827
           |                      |
           |     Point estimate   | [95% Conf. Interval]
           |----------------------+----------------------
    Odds ratio |       .8177419    |  .2997636    2.378989
(exact)
 Prev. frac. ex. |     .1822581    | -1.378989    .7002364
(exact)
 Prev. frac. pop |     .0169542    |
           +-------------------------------------------
                     chi2(1) =     0.18  Pr>chi2 = 0.6684

. gen reduced = r2level ~=0

. sort reduced black

. collapse (sum) count = count, by(reduced black)
```

-EHLA DATA-1

```
. cc black reduced [fw=count]
                                                  Proportion
           |  Exposed  Unexposed |    Total     Exposed
           |  < 75%     >75%     |
-----------+----------------------+----------------------
     Cases |      25        155 |      180      0.1389
  Controls |      37         78 |      115      0.3217
-----------+----------------------+----------------------
     Total |      62        233 |      295      0.2102
           |                     |
           |    Point estimate   | [95% Conf. Interval]
           |---------------------+----------------------
  Odds ratio |       .3400174     | .1828834    .6283923
(exact)
 Prev. frac. ex. |       .6599826     | .3716077    .8171166
(exact)
 Prev. frac. pop |       .2123422     |
           +-------------------------------------------
                     chi2(1) =    14.13  Pr>chi2 = 0.0002
```

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VANDERBILT UNIVERSITY
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August 18, 2003

Dr. Katherine Moore
Department of the Army
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Doctor Moore:

Digna S. Forbes, M.D. is the principal investigator (40% effort) for the subcontract at Meharry Medical College, for the recently funded National Cancer Institute Breast Cancer SPORE Vanderbilt University Medical Center, 08/03-12/08. She will collaborate with me on Project 4 of this grant entitled "Molecular Epidemiology of Proliferative Breast Disease". She will supervise the follow-up work by the study staff at Metro General Hospital/ Hubbard Hospital. Together with Dr. Sanders, she will perform the histologic diagnoses, PCR analyses, and immunohistochemical analyses of the entry biopsies of study subjects. This work will be done under the guidance of Drs. Page, Moses, Parl and myself. They will be assisted by laboratory technicians who are already funded by grants of Drs. Dupont, Moses Parl, and from other sources.

I have worked with Dr. Forbes over the past two years and the African American cohort established through the support of her DOD grant will be used to complete Specific Aim #3 in Project #4 of the SPORE. Dr. Forbes is an outstanding clinical investigator. I am greatly looking forward to collaborating with her on this important study.

Sincerely,

William D. Dupont, PhD
Professor and Chair
Division of Biostatistics

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